FUNCTIONAL AND REGULATORY STUDIES OF CYTOCHROME C OXIDASE ASSEMBLY FACTOR 6

A Thesis

by

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ABSTRACT

The mitochondrial respiratory chain (MRC) consists of four multi-protein complexes in the inner membrane of mitochondria and is the main site of cellular respiration. MRC biogenesis is facilitated by several assembly factors, many of which remain unidentified and uncharacterized. Using an integrative genomic approach, we recently identified an evolutionarily conserved role of a previously uncharacterized mitochondrial protein, Coa6, in the assembly of MRC complex IV, also known as cytochrome c oxidase (CcO). Follow-up experiments showed that the CcO assembly defect could be rescued by copper supplementation, implicating Coa6 in mitochondrial copper metabolism. However, the precise molecular function of Coa6 in CcO assembly remained unknown. To understand the function and regulation of Coa6, we have purified Coa6 to homogeneity and generated a polyclonal antiCoa6 antibody, which was used to study Coa6 regulation under different nutrient, chemical, and genetic perturbations. By measuring Coa6 protein levels under these perturbations, we found that like Cox2, a copper containing subunit of CcO, Coa6 is regulated by copper abundance, further supporting its role in copper delivery to CcO. In order to place Coa6 in the CcO copper delivery pathway, we performed a genetic epistasis analysis and found synthetic lethal interactions between Coa6 and Sco2, a well-known mitochondrial copper metallochaperone, and Coa6 and Cox12, a known subunit of CcO. These results suggest overlapping but non-redundant roles of Coa6, Cox12 and Sco2 in copper delivery to Cox2.

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NOMENCLATURE

CcO	Cytochrome c oxidase
Coa6	Cytochrome c oxidase assembly factor 6
IM	Inner membrane
IMS	Intermembrane space
MRC	Mitochondrial respiratory chain
WT	Wild type

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CHAPTER I

INTRODUCTION

Cytochrome c oxidase (CcO) is the terminal enzyme of the mitochondrial respiratory chain (MRC), a system of four electron-transport complexes present in the inner membrane (IM) of the mitochondria. Together, the four complexes of the MRC generate a proton gradient, which is harnessed by ATP synthase to produce mitochondrial ATP. CcO is an evolutionarily conserved enzyme that consists of 13 polypeptides in addition to several cofactors, including two copper centers (Cu_A and Cu_B), two heme groups (heme a and a_3), and a magnesium and zinc ion (1). The assembly of CcO is highly complex and is not completely understood. To date, approximately 40 CcO assembly factors have been discovered, but for many of them their precise role in CcO biogenesis remains unclear (2). Elucidating the function of these uncharacterized assembly factors is not only crucial for understanding the biogenesis of CcO, but also has biomedical significance, since mutations in several of these CcO assembly factors result in monogenic mitochondrial diseases (3).

Recently, we identified a novel CcO assembly factor, Coa6, as an evolutionarily conserved intermembrane space (IMS) protein essential for assembly of the CcO (4). Studies in our lab using a knockout model in yeast and knockdown models in human cell lines and zebrafish embryos confirmed that Coa6 is essential for CcO assembly and mitochondrial respiration (4). Furthermore, our work and a subsequent study showed

that the mutations reported in human *COA6* (*C1orf31*) are pathogenic and result in mitochondrial disorder characterized by hypertrophic cardiomyopathy (4,5).

Coa6 contains a conserved $Cx_9Cx_nCx_{10}C$ motif, which is similar to a twin Cx_9C motif found in mitochondrial IMS proteins involved in copper delivery to CcO. Both of the reported Coa6 disease patients had mutations within this motif (4-6). Multiple twin Cx_9C motif-containing IMS proteins, such as Cox11, Cox17, Cox19, Cox23, Sco1, Sco2, and Cmc1 have been shown to be involved in copper delivery to CcO (7), but the exact sequence of these proteins in the copper delivery pathway remains unclear. The addition of exogenous copper to growth media is able to rescue the respiratory deficient growth of *cox17*Δ and *cmc1*Δ cells (8,9), and likewise, copper is able to fully restore growth of *coa6*Δ yeast (4). This result strongly implicates Coa6 in copper delivery to CcO, possibly as a metallochaperone. Coa6 has all the features of a CcO copper metallochaperone in that it contains a conserved cysteine-rich motif, it is localized to the IMS, and exogenous copper is able to rescue CcO assembly defect in *coa6*Δ. Despite these observations, however, the exact molecular function of Coa6 remains unknown.

Due to its clinical significance in mitochondrial disease pathogenesis, evolutionary conservation, and lack of assigned function, I have undertaken a detailed study of the function and regulation of Coa6 in the yeast *Saccharomyces cerevisiae*. *S. cerevisiae* serves as a useful model to study Coa6 because three of the four MRC complexes are conserved, which suggests that essential assembly factors are also conserved.

Additionally, yeast are able to proliferate using different carbon sources as fuel to generate ATP by using either glycolysis or mitochondrial respiration. Mitochondrial biogenesis and respiration is stimulated in respiro-fermentative and non-fermentative media containing galactose and glycerol/ethanol, respectively, providing an optimal growth conditions to study the function of a mitochondrial protein (10). Additionally, mitochondrial biogenesis is regulated by the availability of copper to the media, such that transcription of mitochondrial proteins is downregulated under copper limiting conditions (11). Since these nutritional perturbations are well characterized in the yeast, I have been able to study the regulation of Coa6 under these conditions.

Using an antibody against native Coa6 protein, I show that Coa6 is regulated by carbon source, growth phase, and copper availability, further implicating Coa6 in CcO assembly. A genetic interaction study discovered a synthetic lethal interaction between Coa6 and Sco2, a well-known mitochondrial copper metallochaperone, under respiratory growth conditions. I found that simultaneous deletion of Coa6 and Sco2 results in a complete loss of Cox2, thus providing biochemical basis for the synthetic lethal interaction. Interestingly, this phenotype could not be rescued by copper suggesting that these two proteins act in parallel pathway of copper delivery to CcO. Unexpectedly, a similar synthetic lethal interaction was also uncovered between Coa6 and Cox12, one of the core subunits of CcO. Taken together, these results firmly place Coa6 in copper delivery pathway to CcO and suggest an overlapping but non-redundant role of Coa6, Sco2 and Cox12 in CcO biogenesis.

CHAPTER II

PURIFICATION OF COA6

Pure Coa6 is necessary to ascertain copper binding and coordination, determine its structure, identify interacting partners, assign specific biochemical activity and generate antibody that specifically recognizes the native protein in its host organism. Therefore, I purified Coa6 and here I describe the purification scheme used to purify Coa6 for the purpose of antibody generation.

Materials and Methods

Coa6 expression and purification

The yeast *COA6* was first subcloned into a pET28a-His_6-GFP-TEV vector using *EcoRI* and *XhoI* restriction sites. This construct was then transformed into Rosetta DE3 *Escherichia coli* cells to express recombinant Coa6. Briefly, transformed cells were grown in LB medium supplemented with 25 µg/mL chloramphenicol and 50 µg/mL kanamycin at 37^{O}C till A_{600} of 0.5. Protein expression was then induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 16 hours at 18^{O}C. The cells were pelleted at 5,000g for 15 minutes and resuspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, and 10% glycerol) with protease inhibitor cocktail (Roche Diagnostic). The cells were lysed using a sonic dismembrator (Fisher Scientific) and cell

debris were pelleted at 16,000g for 30 minutes at 4^{O}C. The supernatant was passed through a HisTrap™ HP column (GE Healthcare Life Sciences) and washed with 20 column volumes of washing buffer (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 10% glycerol, and 50 mM imidazole). The fused protein was eluted using 5 column volumes of elution buffer (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 10% glycerol, and 500 mM imidazole). The imidazole concentration of the eluent was diluted to 50 mM using lysis buffer immediately prior to the addition of recombinant His_6-Tobacco Etch Virus (TEV) protease at a ratio of 1 mg TEV protease per 4 mg fused protein. TEV-catalyzed digestion of Coa6 fusion protein was performed for 4 hours at room temperature in the presence of 5 mM DTT. The digested sample was then passed through a second HisTrap™ HP column to bind His_6-GFP and TEV. Isolated Coa6 protein was collected and passed through a Superdex 200 10/300 FL column (GE Healthcare Life Sciences) to separate Coa6 from residual His_6-GFP and TEV that failed to bind to the HisTrap™. At each step, protein purity and quantity were assessed by SDSPAGE with Coomassie stain and Bradford assay, respectively.

Generation of Coa6 antibody

1.5 mg of pure Coa6 protein was sent to Rockland™ Antibodies & Assays for antibody generation. Coa6 antibody was purified from rabbit antisera by incubating the sera with Coa6 protein coupled to Affi Gel 10 beads (Biorad) in a 5 mL Qiagen column for 2-4 hours at room temperature. The column was washed using 50-100 mL PBS. Pure

polyclonal antibody was eluted using acid buffer (0.2 M glycine, 500 mM NaCl, pH 2.0) into a conical tube containing 1.5 M Tris (pH 8.8) to neutralize the pH. Purified antibody was aliquoted and stored at -20^{O}C.

Whole cell yeast protein extraction

Yeast cells were cultured at 30°C in 7.5 mL of YPGE (1% yeast extract, 2% bactopeptone, 3% glycerol and 1% ethanol) till mid-logarithmic phase and were pelleted by centrifugation at 3,000g for 5 minutes at 4°C. The cell pellet (~75 mg) was washed with water and resuspended in 350 µL SUMEB buffer (10 mM MOPS, pH 6.8, 8 M urea, 10 mM EDTA, 1.0% SDS) containing 1 mM PMSF and protease inhibitor cocktail (Roche Diagnostic) and transferred to a 2 mL tube containing 350 mg of acid-washed glass beads (Sigma-Aldrich). Cells were vortexted for 1 minute followed by 30 seconds on ice. This procedure was repeated three times. Lysed cells were kept on ice for 10 minutes to reduce bubbles and then transferred to 70^{O}C for 10 minutes. Cell debris and glass beads were spun down at 14,000g for 10 minutes at 4^{O}C. The supernatant was transferred to a separate tube and protein was measured by BCA assay (Life Technologies).

SDSPAGE and Western blotting

50 µg of yeast whole cell protein lysate was separated on 12% Bis-Tris NUPAGE gels

(Life Technologies) and blotted onto a polyvinylidene difluoride (PVDF) membrane. Membranes were blocked for 1 hour in 5% nonfat milk dissolved in Tris-buffered saline with 0.1% Tween20 (TBST-milk). Membranes were incubated with primary antibody in TBST-milk overnight at 4°C. Primary antibodies were used at the following dilutions: Coa6, 1:1,000; Porin, 1:50,000 (Abcam 110326). Secondary antibodies were used at a 1:5,000 dilution in TBST-milk for 1 hour at room temperature. Membranes were developed using WesternLightning Plus-ECL (PerkinElmer) or SuperSignal West Femto (Thermo Scientific).

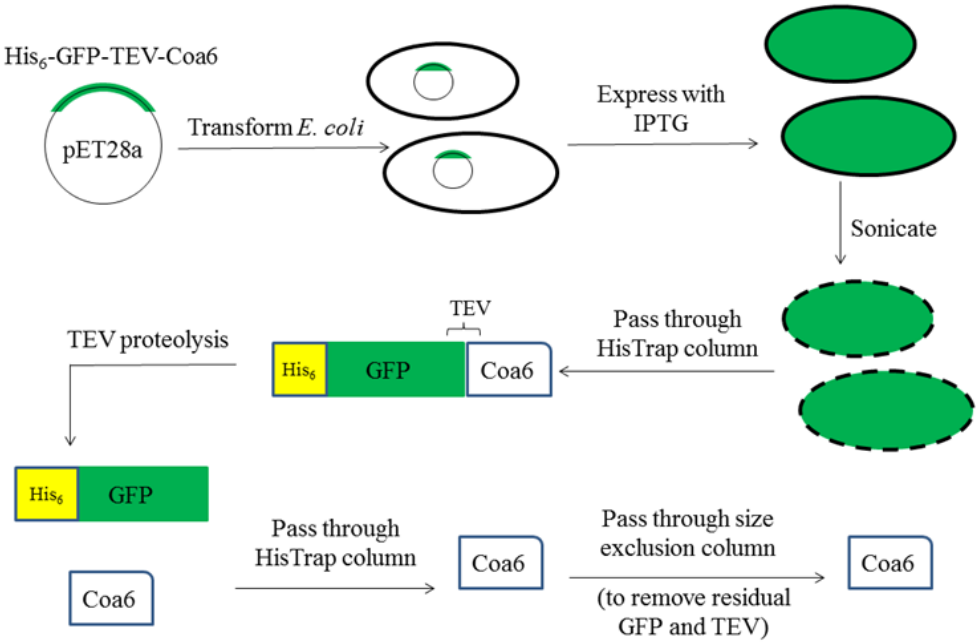

Figure 1. Coa6 purification scheme.

Results

Purification of Coa6

Coa6 with a His_6-GFP-TEV tag and Rosetta DE3 expression system were chosen for large-scale protein purification as described in Materials and Methods and illustrated in Fig. 1. GFP increased the solubility of the fusion construct and provided a visual means

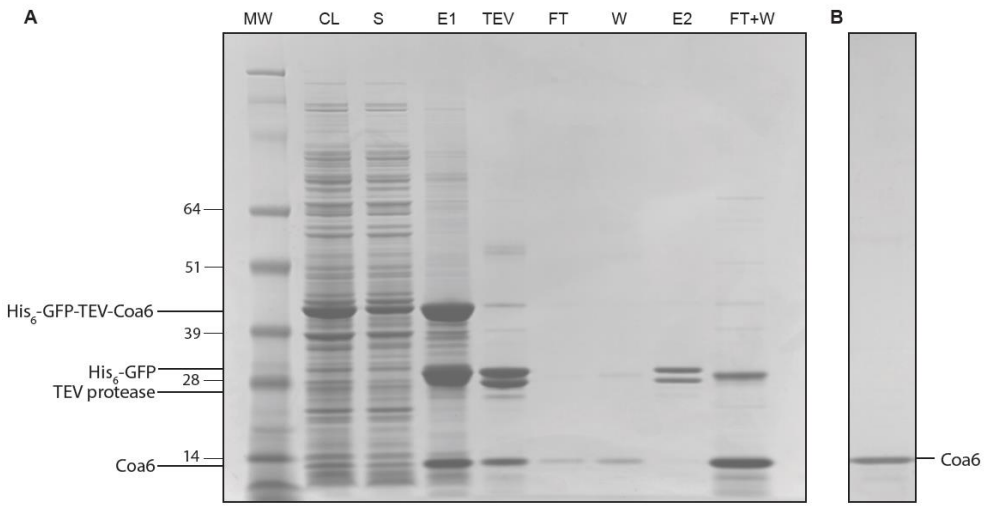

Figure 2. Purification of Coa6 from *E.coli*. (A) *E.coli* Rosetta DE3 cells overexpressing a fusion protein His_6-GFP-TEV-Coa6 were sonicated (CL) and centrifuged to pellet cellular debris. The supernatant (S) was passed through a HisTrap™ HP column and the purified fraction (E1) was subjected to TEV proteolysis for 4 hours at room temperature (TEV). The solution was passed through a second HisTrap™ HP column. Both the flow through (FT) and wash (W) were collected. Elution of the column revealed all Coa6 had eluted in the flow through and wash (E2). The flow through and wash were pooled together and concentrated to ~2 mL (FT+W). (B) Purified Coa6 after passing through Superdex 200 10/300 FL column. Molecular weight (MW) markers (in kilodaltons) and the positions of individual proteins are indicated.

of determining expression and location of the fusion protein throughout the purification process. Protease inhibitors were present during cell lysis to prevent premature cleavage of the fusion protein, but cleaved His_6-GFP and Coa6 can nevertheless be detected following elution from the first HisTrap™ HP column (Fig. 2A, lane E1). Addition of TEV protease cleaved the fusion protein, releasing Coa6 and allowing it to pass through a second HisTrap™ HP column in the flow through and wash (Fig. 2A, lanes FT and W). When both fractions were combined and concentrated, residual TEV protease was detected (Fig.2A, lane FT+W). Therefore, the flow through + wash was subjected to size exclusion chromatography by injecting onto a Superdex 200 10/300 FL column to remove these residual contaminants (Fig. 2B). A total yield of 1 mg pure Coa6 per 1 L of bacterial culture was obtained.

Purified Coa6 antibody specifically recognizes yeast Coa6

Polyclonal antibody was produced and purified from rabbit sera as explained in Materials and Methods. To confirm the specificity of our antibody, I probed for Coa6 levels in yeast wild type (WT), *coa6*Δ, and *coa6*Δ transformed with a multi-copy pRS426 plasmid expressing *COA6*. Cells were grown in YPGE to mid-logarithmic growth phase. Coa6 knockout cells were supplemented with 5 µM copper chloride ($CuCl_2$) to allow for equivalent growth. I observed a band in WT cells at ~12 kDa, which corresponds with the molecular weight of Coa6 (Fig. 3). The same band was absent in *coa6*Δ cells, but was present at a higher intensity in the same position in cells

overexpressing native Coa6, confirming the identity of Coa6. The lack of other additional high-intensity bands suggests high specificity of our antibody, making it a useful resource to study regulation of Coa6 in yeast cells (Fig. 3).

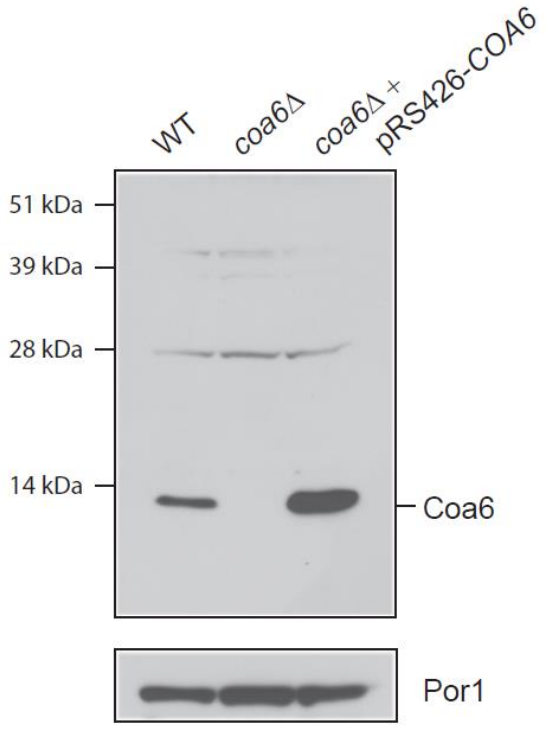

Figure 3. Validation of Coa6 antibody. Yeast whole cell protein was extracted from WT, *coa6*Δ, and *coa6*Δ transformed with pRS426-*COA6* grown in YPGE liquid medium to mid-logarithmic growth phase. *coa6*Δ cells were grown in the presence of 5 µM $CuCl_2$. 50 µg of whole cell protein was loaded on a 12% Bis-Tris gel and analyzed using SDSPAGE/Western blot. Top panel: Western blot using purified polyclonal Coa6 antibody. The position of Coa6 is indicated. Bottom panel: Por1 was used as a loading control.

CHAPTER III

ANALYSIS OF COA6 REGULATION AND FUNCTION

In order to further study the role of Coa6 in copper transport to CcO, it is necessary to identify the growth conditions and factors that regulate Coa6 levels in yeast cells. Identifying Coa6 regulatory factors will not only provide valuable insights into its function but will also allow us to optimize future experiments directed at analyzing Coa6 function in vivo by determining when Coa6 is most abundant. With this goal, we tested the effect of different growth conditions, nutrients, copper abundance and the presence of mitochondrial CcO assembly factors on Coa6 expression.

Materials and Methods

Yeast strains and culture conditions

All strains used were obtained from Open Biosystems or independently created in the Gohil lab (Table 1). For growth in liquid medium, yeast cells were precultured in YPD (1% yeast extract, 2% peptone, and 2% glucose) at 30°C and later inoculated into YPD media or media containing 2% galactose (YPGal) or 3% glycerol + 1% ethanol (YPGE). Growth measurements were done either by measuring optical density of liquid growth cultures using a DU® 730 UV/Vis Spectrophotometer (Beckman Coulter) or by seeding serially diluted cells on solid medium. The cell number of overnight precultures was

calculated with a hemocytometer prior to being serially diluted at 10^4, 10^3, 10^2, and 10^1 cells per 3 μl. Dilutions were spotted onto plates containing different media as indicated and incubated at 30°C for 3 days.

Table 1.
Yeast strains used in this study.

Strain	Genotype	Source
BY4742 WT	MAT α his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0	Open Biosystems
BY4742 *coa6*Δ	Mat α, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, *coa6*Δ:: NatMX4	Gohil lab
BY4741 WT	Mat a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0	Open Biosystems
*coa6*Δ	Mat a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0, *coa6*Δ:: KanMX4	Open Biosystems
*sco1*Δ	Mat a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0, *sco1*Δ::KanMX4	Open Biosystems
*sco2*Δ	Mat a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0, *sco2*Δ::KanMX4	Open Biosystems
*cox11*Δ	Mat a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0, *cox11*Δ::KanMX4	Open Biosystems
*cox12*Δ	Mat a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0, *cox12*Δ::KanMX4	Open Biosystems
*cox17*Δ	Mat a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0, *cox17*Δ:: HphMX4	Gohil lab
*cox19*Δ	Mat a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0, *cox19*Δ::KanMX4	Open Biosystems
*cox23*Δ	Mat a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0, *cox23*Δ::KanMX4	Open Biosystems
*cmc1*Δ	Mat a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0, *cmc1*Δ::KanMX4	Open Biosystems
*cmc2*Δ	Mat a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0, *cmc2*Δ::KanMX4	Open Biosystems
*cmc3*Δ	Mat a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0, *cmc3*Δ::KanMX4	Open Biosystems
STY3 (*cox12*Δ*coa6*Δ)	Mat a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0, lys200, met1500, *cox12*Δ::KanMX4, *coa6*Δ::clonNAT	Gohil lab
STY10 *(sco2*Δ*coa6*Δ*)*	Mat a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0, *sco2*Δ::KanMX4, *coa6*Δ::clonNAT	Gohil lab

Whole cell yeast protein extraction and isolation of mitochondrial fractions

Yeast cells (60-80 mg wet weight) were suspended in 350 µL SUMEB buffer (10 mM MOPS, pH 6.8, 8 M urea, 10 mM EDTA, 1.0% SDS) containing 1 mM PMSF and protease inhibitor cocktail (Roche Diagnostic). Cell were transferred to a fresh tube containing 350 mg of acid-washed glass beads (Sigma-Aldrich) and were vortexted three times for 1 minute each, with 30 seconds incubation on ice between every vortex step. Lysed cells were kept on ice for 10 minutes to reduce bubbles and then heated to 70^{O}C for 10 minutes. Cell debris and glass beads were spun down at 14,000g for 10 minutes at 4^{O}C. The supernatant was transferred to a separate tube and protein was quantified by BCA assay (Life Technologies). Mitochondria were isolated from yeast cells grown to early stationary growth phase in YPD by a previously described method (12).

SDSPAGE and Western blotting

Whole cell yeast protein lysate was fractionated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDSPAGE) using precast 12% Bis-Tris gels and blotted onto a polyvinylidene difluoride (PVDF) membrane. Membranes were blocked for 1 hour in 5% nonfat milk dissolved in Tris-buffered saline with 0.1% Tween20 (TBST-milk), followed by incubation with primary antibody in TBST-milk overnight at 4^{o}C. Primary antibodies were used at the following dilutions: Coa6, 1:1,000; Cox1, 1:10,000 (Abcam 110270); Cox2, 1:10,000 (Abcam 110271); Cox17, 1:250 (from Dr.

Alexander Tzagoloff); Cmc1, 1:500 (from Dr. Antoni Barrientos); HA, 1:10,000 (Sigma H9658); Porin, 1:50,000 (Abcam 110326); Pgk1, 1:50,000 (Life Technologies 459250).

Results

Coa6 is regulated by mitochondrial biogenesis factors

Previous studies have shown that growth phase and carbon source regulate

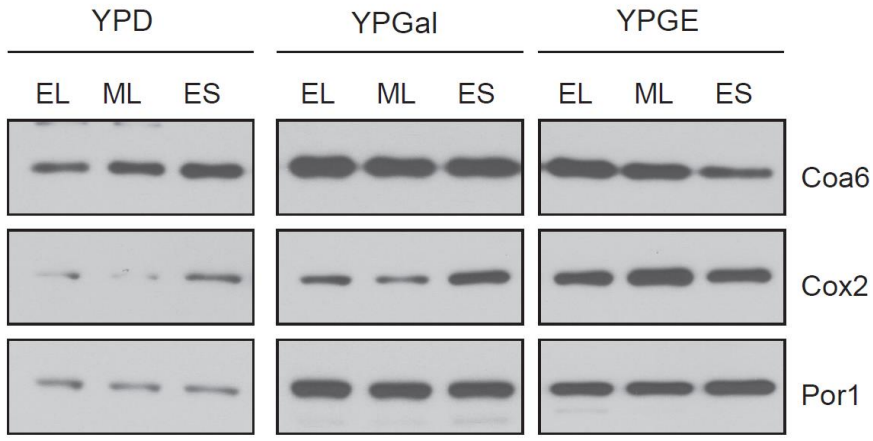

Figure 4. Coa6 is regulated by mitochondrial biogenesis factors. WT yeast were precultured in YPD and inoculated in fresh YPD, YPGal, or YPGE liquid media at 30°C with a starting A_{600} of 0.1. Cells were grown to early logarithmic (EL), mid-logarithmic (ML), and early stationary (ES) growth phase in each media and whole cell protein was extracted. Coa6 was analyzed alongside Cox2 by Western blotting. Porin (Por1) was used as a loading control.

mitochondrial biogenesis (10,13). Therefore, I hypothesized that Coa6, being a mitochondrial protein required for MRC complex IV biogenesis, could be regulated by factors that promote mitochondrial biogenesis. To test this hypothesis, steady state levels of Coa6 were determined in WT yeast cells grown in fermentable (YPD), respiro-fermentable (YPGal), or non-fermentable (YPGE) liquid media at early logarithmic (EL), mid-logarithmic (ML), or early stationary (ES) growth phases. As seen in Fig. 4, Coa6 levels were highest in respiro-fermentable and non-fermentable media, indicating Coa6 is regulated by carbon source. Additionally, expression levels of Coa6 increase with growth phase in fermentable media, but decrease in non-fermentable media, demonstrating that Coa6 levels depend on growth phase. Interestingly, increase in Coa6 levels precedes those of Cox2, one of the copper containing subunits of CcO, suggesting that Coa6 must be present for optimal expression of Cox2.

Coa6 levels decrease in response to copper deprivation

A recent study has shown that iron-containing proteins of the MRC, specifically MRC complex I, MRC complex III, and cytochrome c, decrease in response to increasing amounts of iron chelator deferoxamine (DFO) in mouse muscle cells (14). Since Coa6 has been implicated in the copper delivery pathway to CcO, I hypothesized that Coa6 levels would alter in response to copper chelation and supplementation. In order to identify optimal concentration of copper-specific chelator bathocuproinedisulfonic acid (BCS) and copper, WT yeast were grown in YPGE and YPGal with increasing amounts

of BCS or copper chloride ($CuCl_2$). An increasing amount of BCS is expected to deprive yeast cells of bioavailable form of copper and therefore would reduce copper containing MRC complex IV, leading to diminished growth in conditions that require mitochondrial ATP synthesis. As expected, increasing amounts of BCS severely reduced yeast growth in YPGE media, whereas additional copper supplementation to the media promoted yeast growth (Fig. 5A). A similar but less severe pattern can be seen when cells are

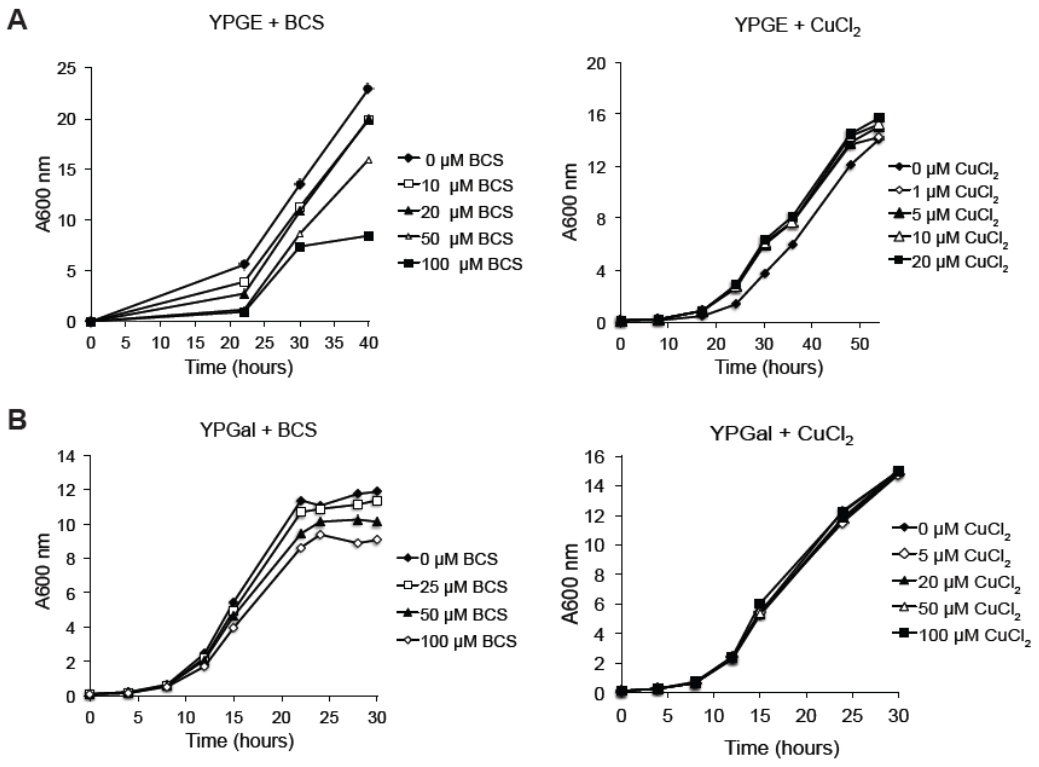

Figure 5. Copper deprivation retards yeast growth under respiratory growth conditions. WT yeast were precultured in YPD and inoculated in either fresh (A) YPGE or (B) YPGal liquid media with increasing amounts of BCS and $CuCl_2$ at 30°C with a starting A_{600} of 0.1. Growth was analyzed by measuring absorbance at 600 nm.

grown in a respiro-fermentative conditions in YPGal medium (Fig. 5B). Therefore, in order to work with equivalent amounts of cells, I chose to probe for Coa6 levels in YPGal media supplemented with BCS and $CuCl_2$. Coa6 levels decreased with increasing BCS addition, whereas copper supplementation appears to increase Coa6 levels (Fig. 6A). In a similar fashion, Cox2 levels decrease drastically under copper limiting conditions, while copper supplementation slightly increases Cox2 (Fig. 6A). Therefore, Coa6 and Cox2 follow a similar pattern in response to altering levels of copper in the environment. In contrast, Cox17, a known metallochaperone that transfers copper to Cox11 and Sco1 (15), remains constant regardless of copper availability in the media

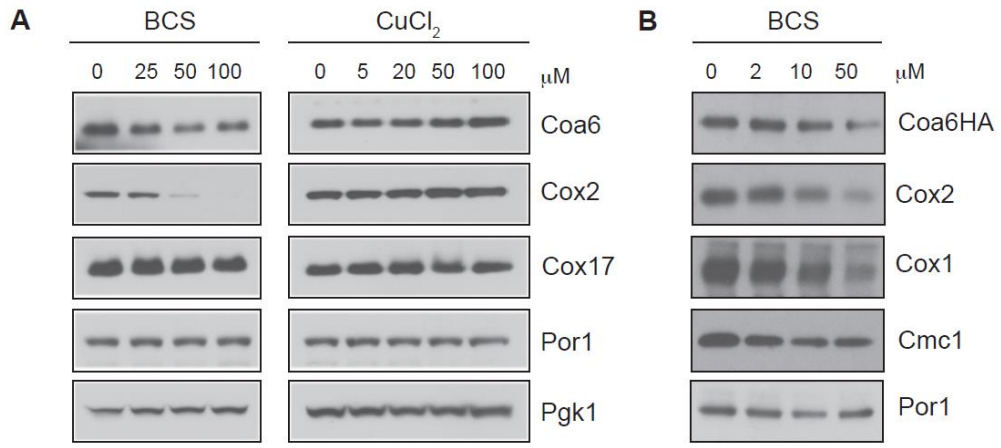

Figure 6. Coa6 levels are dependent on copper availability. (A) Western blot analysis of whole cell protein lysate prepared from WT yeast cells grown to mid-logarithmic growth phase in YPGal medium with increasing amounts of BCS or $CuCl_2$.(B) Western blot analysis of mitochondria isolated from WT cells grown in YPD to early stationary growth phase with increasing amounts of BCS. Por1 and Pgk1 were used as loading controls.

(Fig. 6A). The decrease in Coa6 with BCS supplementation is more obvious in mitochondrial samples isolated from chromosomally tagged-Coa6 cells (Fig. 6B). As expected, Cox1 and Cox2 the copper containing subunits of CcO, also decreases with increasing BCS (Fig. 6B). Interestingly, Cmc1, a copper-binding CcO assembly factor, also decreases with increasing amounts of BCS in isolated mitochondria (Fig. 6B).

Coa6 expression is independent of other IMS CcO assembly factors

Previous reports (4,9) have shown that exogenous copper supplementation can overcome the deletion of CcO assembly factors, like Coa6, Cox17, and Cmc1, suggesting that parallel pathways of copper delivery to CcO exist, and that the protein of one pathway

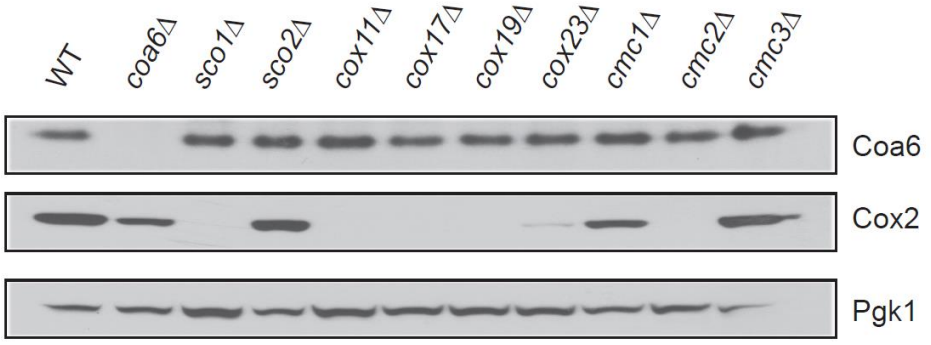

Figure 7. Coa6 expression is independent of mitochondrial CcO assembly factors. Whole cell lysate protein was extracted from the indicated knockout strains grown in YPGal liquid medium to mid-logarithmic growth phase and analyzed by Western blotting with indicated antibodies.

may compensate for the lack of the other. For example, Cmc1 levels increase significantly in response to deletion of Cmc2, which suggests they share a common function (16). To explore the possibility that Coa6 levels may be regulated by other CcO assembly factors implicated in copper delivery pathway, I probed for Coa6 levels in various knockout strains of known copper metallochaperones (Cox11, Cox17, Cox19, Cox23, Sco1, and Sco2 (7)) and twin Cx_9C motif containing proteins implicated in CcO assembly (Cmc1 (9), Cmc2 (16), and Cmc3 (17)). Overall, Coa6 levels remain constant despite the deletion of the CcO assembly factors tested (Fig. 7), suggesting that Coa6 abundance is independent of the presence of other CcO assembly factors.

Coa6 works in conjunction with Sco2 and Cox12 to maintain Cox2 levels

In order to place Coa6 in the CcO copper delivery pathway, a genetic epistasis study was performed, where *coa6*Δ cells were crossed with the deletion strains of each of the known copper metallochaperones and Cx_9C proteins implicated in CcO assembly. The resulting double knockouts were extensively phenotyped in different growth media, temperatures, and copper supplementations. Analysis of the growth phenotypes of both the parent knockouts and double knockout revealed that *coa6*Δ*sco2*Δ is synthetic lethal when grown on non-fermentable media (YPGE) (Fig. 8A). Additionally, *coa6*Δ*cox12*Δ is also synthetic lethal when grown on complete synthetic respiro-fermentable media (CS Galactose) (Fig. 8B). Exogenous copper fails to rescue the growth defect of either double knockout (Fig. 8A-B) suggesting that these proteins are acting in parallel pathways. Sco2 is a mitochondrial

inner membrane bound copper metallochaperone that extends into the IMS and has been suggested to act as an oxidoreductase in human cells (18,19), while Cox12 is a subunit of CcO itself whose biochemical function is unknown (20). The synthetic lethal interaction of Coa6 with Sco2 is not surprising since prior evidence for their involvement in CcO copper

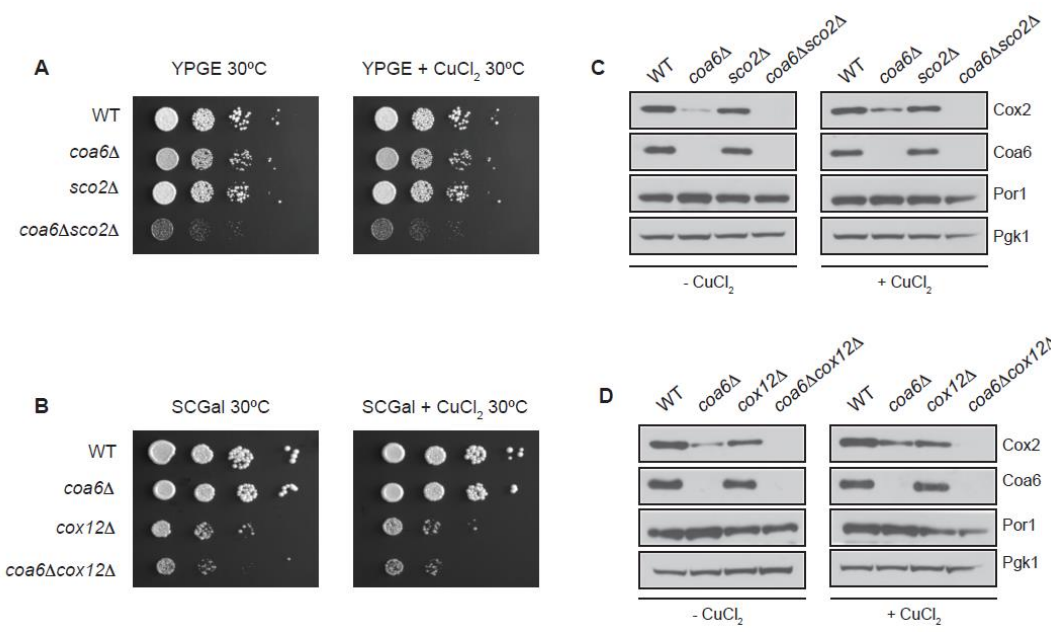

Figure 8. Coa6, Sco2 and Cox12 have overlapping functions in CcO assembly. (A) Serially diluted WT, *coa6*Δ, *sco2*Δ, and *coa6*Δ*sco2*Δ cells were spotted on YPGE plates with and without 5 µM $CuCl_2$ at 30°C. (B) Serially diluted WT, *coa6*Δ, *cox12*Δ, and *coa6*Δ*cox12*Δ cells were spotted on synthetic complete (SC) galactose with and without 5 µM $CuCl_2$ at 30°C. (C-D) WT, single knockouts of Coa6, Sco2, Cox12, and *coa6*Δ*sco2*Δ and *coa6*Δ*cox12*Δ double knockouts were grown to mid-logarithmic phase in YPGal liquid medium with and without 5 µM $CuCl_2$ at 30°C. Protein was extracted from whole cells and analyzed by Western blot. Por1 and Pgk1 were used as loading controls.

delivery pathway was available, but the synthetic lethal interaction between Coa6 and Cox12 is surprising, and it suggests a hitherto undiscovered role of Cox12 in copper delivery to CcO subunits. To probe for the mechanism that leads to synthetic lethality of double knockouts, I analyzed levels of the copper containing CcO subunit Cox2. As seen in Fig. 8C-D, Cox2 levels are decreased in *coa6*Δ, *sco2*Δ, and *cox12*Δ to different degrees but in both *coa6*Δ*sco2*Δ and *coa6*Δ*cox12*Δ, Cox2 is completely absent. Exogenous copper fails to restore Cox2 levels in both double knockouts (Fig. 8C-D). Coupled with the growth data, the lack of Cox2 in *coa6*Δ*sco2*Δ and *coa6*Δ*cox12*Δ suggests that Coa6 has an overlapping function with Cox12 and Sco2 to maintain Cox2 levels.

CHAPTER IV

SUMMARY AND CONCLUSIONS

Recent studies in our lab identified Coa6 as an evolutionarily conserved CcO assembly factor in yeast, zebrafish, and humans. Coa6 sequence features, submitochondrial localization and metal supplementation experimentation suggested its role in mitochondrial copper metabolism; however, its precise role in CcO copper delivery pathway remained unknown. In an effort to uncover its function in CcO assembly, I present the first purification scheme for yeast Coa6 protein. Purified Coa6 was used to generate a polyclonal antibody, which I employed to study its regulation and found that Coa6 is regulated by carbon source, growth phase, and copper abundance, all of which implicated Coa6 in the mitochondrial copper delivery pathway to CcO. A genetic epistasis experiment placed Coa6 in the copper delivery pathway and suggested its overlapping function with Sco2, a well-known mitochondrial copper metallochaperone, and Cox12, a subunit of CcO, in maintaining steady state levels of Cox2.

Because Coa6 is a conserved mitochondrial protein, I first chose to explore if mitochondrial biogenesis factors regulate the expression of Coa6. In *S. cerevisiae*, mitochondrial biogenesis is stimulated in galactose-containing respiro-fermentable and glycerol/ethanol-containing non-fermentable media, while glucose-containing fermentable media causes repression of mitochondrial genes (21). Consistent with this observation, I observe that Coa6 levels are higher in respiro-fermentable and non-

fermentable media than in fermentable media (Fig. 4), indicating Coa6 is regulated by carbon source. Likewise, Coa6 displays growth phase dependent regulation, which is most obvious in fermentable media, where glucose-mediated catabolite repression is released when glucose is consumed by proliferating cells, thereby resulting in derepression of mitochondrial genes (including Coa6). Interestingly, in each growth condition, increase in Coa6 levels precedes Cox2, suggesting that Coa6 must be present for Cox2 expression (Fig. 4). This observation further supports Coa6's role as a CcO assembly factor.

When WT cells are grown under copper limiting conditions through the addition of BCS, a decrease in Coa6 levels can be observed (Fig. 6A). Interestingly, CcO assembly factor Cmc1 also follows a similar pattern under copper deprivation conditions (Fig. 6B). Deprivation of copper has the most severe impact on the stability of the copper containing subunits of CcO, Cox1 and Cox2, both of which need copper to be catalytically active and stably expressed (Fig. 6A-B). A larger decrease in Cox proteins and a smaller decrease in the levels of Coa6 can be possibly explained by a scenario in which some fraction of Coa6 molecules interacts with Cox2 during Cox2 maturation, but the complex is depleted when copper is limiting. Thus, a pool of Cox2-bound Coa6 will deplete in copper limiting condition but unbound Coa6 may remain stable. The same pattern can explain why Cmc1 levels decrease alongside Cox1, as Cmc1 is essential for the synthesis of Cox1 and is part of a high molecular weight complex (9), containing Cox1 (22). Additionally, this model explains why Cox17 levels remain unchanged

despite copper fluctuations, since Cox17 itself has not been shown to directly interact with any of the copper-containing CcO subunits (Fig. 6A).

Exogenous copper supplementation rescues the deletion of Coa6, Cox17, and Cmc1, suggesting the possibility that, in contrast to a single, linear pathway of copper delivery to CcO, parallel pathways of copper delivery to CcO exist. Therefore, it is likely that the proteins involved in these pathways are reciprocally regulated. For example, Cmc1 levels increase when Cmc2 is deleted, indicating they likely have an overlapping function (16). However, unlike Cmc1, Coa6 levels are independent of CcO copper metallochaperones (Fig. 7). While it is possible that one of these proteins overexpresses to compensate for deletion of Coa6, the lack of available antibodies for these proteins makes testing this possibility difficult.

While regulatory studies shed light on the putative function of Coa6, genetic interaction studies can provide more direct insight into Coa6's function. Therefore, a genetic interaction study of Coa6 was performed, which uncovered interactions of Coa6 with both Sco2 and Cox12 (Fig. 8A-B). These results are strengthened by the fact that Cox2 levels are completely absent in both double knockouts (Fig. 8C-D), confirming an overlapping function of Coa6, Sco2 and Cox12 in Cox2 maturation. While the molecular function of Cox12 in CcO biogenesis is not established, mammalian Sco2 has been shown to be required for copper metalation of Cox2 (18). These results taken together in context of our findings suggest that Coa6, Sco2 and Cox12 play a critical role in the

copper delivery pathway to CcO. It would be interesting to see as to what extent these proteins share overlapping functions. This can be tested by checking if *coa6*Δ growth defects can be rescued by overexpression of either Sco2 or Cox12 or both.

The biomedical significance of Coa6 has come to light in recent years with the report of two separate patients with pathogenic mutations in Coa6 (5,6). Without an ascribed function for Coa6, it is extremely difficult to treat diseases that arise from Coa6 mutations, and both reported patients succumbed to hypertrophic cardiomyopathy. While copper supplementation offers an exciting therapuetic option for mitochondrial disease patients with mutations in Coa6, many important questions remain about the mechanism of this rescue. For instance, what role does Coa6 play in copper delivery to CcO? How does Coa6 function alongside Sco2 and Cox12 to maintain mature levels of Cox2? Through the purification of Coa6, generation of native antibody, and uncovering the regulation of Coa6, I have taken important strides in revealing the exact role of Coa6 in the copper delivery pathway for CcO assembly that will guide future experiments to address these questions.

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